

1 **5-HT orchestrates Histone Serotonylation and Citrullination to**
2 **Drive Neutrophil Extracellular Traps and Liver Metastasis**

3 **Authors:** Kaiyuan Liu^{1#}, Yingchao Zhang^{1#}, Genyu Du¹, Xinyu Chen¹, Lingling Xiao²,
4 Luyao Jiang¹, Na Jing¹, Penghui Xu³, Chaoxian Zhao¹, Yiyun Liu¹, Huifang Zhao¹,
5 Yujiao Sun¹, Jinming Wang¹, Chaping Cheng¹, Deng Wang³, Jiahua Pan¹, Wei Xue¹,
6 Pengcheng Zhang⁴, Zhi-Gang Zhang¹, Wei-Qiang Gao^{1,3}, Shu-Heng Jiang¹, Kai
7 Zhang^{1*}, and Helen He Zhu^{1*}

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9 **Affiliations:**

10 ¹State Key Laboratory of Systems Medicine for Cancer, Renji-Med-X Stem Cell Research
11 Center, Department of Urology, Renji Hospital, Shanghai Cancer Institute, Shanghai Jiao
12 Tong University School of Medicine, Shanghai, China;

13 ²Department of Emergency Medicine, Shanghai Seventh People's Hospital, Seventh
14 People's Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai,
15 China;

16 ³Med-X Research Institute, School of Biomedical Engineering, Shanghai Jiao Tong
17 University, Shanghai, China;

18 ⁴School of Biomedical Engineering, Shanghai Tech University, Shanghai, China.

19 # These two authors contribute equally to this paper.

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21 **Correspondence*:** Helen He Zhu (zhuhecrane@shsmu.edu.cn), and Kai Zhang
22 (zhangkaishida@126.com), Tel: 86-21-62932049, Fax: 86-21-68383916.

23 Address: Renji Hospital, 160 Pujian Rd., Shanghai, 200127, China.

26 **Abstract**

27 Serotonin (5-HT) is a neurotransmitter that has been linked to tumorigenesis.
28 Whether and how 5-HT modulates cells in the microenvironment to regulate tumor
29 metastasis remains to be largely unknown. Here, we demonstrate that 5-HT is secreted
30 by neuroendocrine prostate cancer (NEPC) cells to communicate with neutrophils and
31 to induce neutrophil extracellular traps (NETs) in the liver, which in turn facilitates the
32 recruitment of disseminated cancer cells and promotes liver metastasis. 5-HT induces
33 histone seronylation (H3Q5ser) and orchestrates histone citrullination (H3cit) in
34 neutrophils to trigger chromatin decondensation and facilitate the formation of NETs.
35 Interestingly, we uncover in this process a reciprocally reinforcing effect between
36 H3Q5ser and H3cit and a crosstalk between the respective writers TGM2 and PAD4.
37 Genetic ablation or pharmacological targeting of TGM2, or inhibiting 5-HT transporter
38 (SERT) with the FDA-approved antidepressant drug fluoxetine reduces H3Q5ser and
39 H3cit modifications, suppresses NETs formation, and effectively inhibits NEPC, small
40 cell lung cancer, and thyroid medullary cancer liver metastasis. Collectively, the 5-HT-
41 triggered NETs production highlights a targetable neurotransmitter-immune axis in
42 driving liver metastasis of neuroendocrine cancers.

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51 **Introduction**

52 Apart from the classical role of neurotransmitters in neuronal activity, they have
53 long been implicated in the pathological process of different tumor types (1). Serotonin
54 (5-HT) is a neurotransmitter that is positively involved in cancer development by
55 promoting cancer cell proliferation and invasion (2-4). The precise manner in which 5-
56 HT modulates the tumor microenvironment to regulate metastasis remains largely
57 unknown. Recently, it has been reported that 5-HT can be taken up into cells via the
58 serotonin transporter (SERT) and be covalently added to the glutamine residue of target
59 proteins such as small guanosine triphosphate hydrolases (GTPase) and
60 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by the enzyme
61 transglutaminase 2 (TGM2) to regulate platelet granule release and CD8⁺ T cell activity,
62 respectively (5-7). Whether 5-HT plays a role in innate immune cells from the tumor
63 microenvironment to regulate cancer progression remains to be elucidated.

64 Neutrophils are the most abundant cell type in innate immunity. In response to
65 pathogen invasion, neutrophil formed neutrophil extracellular traps (NETs), web-like
66 structures composed of decondensed chromatin and antimicrobial proteins, to kill
67 pathogens (8). Intriguingly, recent studies in cancers suggested that NETs act to
68 promote metastasis by attracting circulating tumor cells or activating dormant cancer
69 cells. Neutrophil chromatin decondensation is a required process for NETs formation
70 (9-11). Seronylation in the 5th glutamine residue of histone H3 (H3Q5ser) has been
71 recently found to participate in chromatin dynamics regulation to activate a permissive
72 transcriptional state (6). Therefore, we hypothesized that 5-HT and H3Q5ser may play
73 a role in NETs formation. Crosstalk among different histone modifications has been
74 recently demonstrated to be essential in the intricate regulation of chromatin dynamics
75 (12). Histone citrullination (H3cit) catalyzed by PAD4 is reported to be critical for

76 chromatin decondensation in NETs (13). Whether H3cit interacts with other histone
77 modification such as H3Q5ser in the process of NETs formation is an interesting but
78 unexplored question.

79 Metastasis is the major cause of death in prostate cancer (PCa) (14, 15).
80 Accumulating evidence shows that the liver, a severely understudied target organ in
81 PCa, is frequently colonized by advanced PCa, especially by the most lethal
82 neuroendocrine prostate cancer (NEPC) variant with a rate of more than 45% (16, 17).
83 These patients with liver metastases had the worst median overall survival (18). We
84 have previously generated a murine NEPC orthotopic implantation model with a high
85 penetrance of liver metastasis (19). Accumulation of neutrophils in the liver of this
86 NEPC tumor-bearing mouse model was interestingly found at early stage of liver
87 metastasis (19). NEPC displays features of neuroendocrine cells including the
88 production of neuroactive peptides and neurotransmitters such as 5-HT (2, 20, 21). We
89 asked whether disseminated NEPC communicates with neutrophils in the liver via 5-
90 HT to shape a metastasis-favorable microenvironment.

91 Here, we identified that 5-HT induces TGM2-mediated H3Q5ser and enhances
92 PAD4-catalyzed H3cit to drive NETs formation. NEPC-derived 5-HT promotes the
93 prostate-to-liver metastasis by crosstalk with hepatic neutrophils.

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101 **Results**

102 **Neutrophil infiltration and NETs formation at early stage promote NEPC liver**
103 **metastasis.**

104 To investigate how immune microenvironment regulates metastasis in the liver, we
105 analyzed immune cell profile in NEPC liver metastasis via a multi-omics-based
106 computational tool, Multi-omics Immuno-Oncology Biological Research (IOBR), on a
107 previously reported Stand-Up-to-Cancer (SU2C) dataset (22-24). As shown in [Figure](#)
108 [1A](#), neutrophils were the most abundant immune cells in liver metastasis of PCa
109 patients. We then compared the neutrophil enrichment among different PCa metastatic
110 sites using the SU2C dataset, and found a predominant neutrophil infiltration in liver
111 metastasis compared to metastatic lesions in other distant sites ([Figure 1B](#)). We
112 previously developed a NEPC liver metastasis mouse model, in which, the
113 *PbCre⁺;Rb1^{ff};Trp53^{ff} (Rb1^{Δ/Δ}Trp53^{Δ/Δ})* tumor organoid was inoculated into the prostate
114 of wild type (WT) C57BL/6 mice (19). Consistently, flow cytometric analysis on this
115 model revealed that Cd11b⁺Ly6G⁺ neutrophils accumulated in livers with micro-
116 metastases less than 200 μm in diameter, suggesting an increase of neutrophils at the
117 early stage of liver metastasis ([Figures 1, C and D](#)).

118 The production of neutrophil extracellular traps (NETs) is an important function of
119 neutrophils. We performed immunohistochemical (IHC) staining against a well-
120 established neuroendocrine differentiation marker, the neural cell adhesion molecule-1
121 (Ncam1), and conducted immunofluorescence (IF) co-staining against
122 myeloperoxidase (MPO), a neutrophil marker, and histone H3 citrullination (H3cit), a
123 NETs marker, on serial sections of normal WT liver and liver metastases from the
124 *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* organoid-implanted mice ([Figure 1E](#)). Quantification of H3cit and MPO
125 double positive cell frequency showed significantly increased NETs formation from

126 micro to macro liver metastases of *Rbl^{Δ/Δ}Trp53^{Δ/Δ}* NEPC tumor-bearing mice in
127 contrast to normal liver samples from WT mice (Figure 1F).

128 Next, we determined the NETs formation in both primary prostate tumors and liver
129 metastases in NEPC patients. As shown in Supplemental Figure 1, A and B, CD66⁺
130 neutrophil-derived NETs (H3cit⁺) were much more enriched in liver metastases
131 compared to primary sites. To determine whether hepatic NETs contributed to NEPC
132 metastasis, we injected DNase I to digest NETs in *Rbl^{Δ/Δ}Trp53^{Δ/Δ}* NEPC tumor-bearing
133 mice. Treatment of DNase I degraded NETs formation (Figure 1, G and H.), and
134 significantly reduced number of liver metastatic foci (Figure 1, I and J.). Notably,
135 DNase I treatment significantly prolonged the survival of *Rbl^{Δ/Δ}Trp53^{Δ/Δ}* NEPC tumor-
136 bearing mice compared to vehicle control (Figure 1K). Next, we investigated the impact
137 of NETs on the attraction and retention of NEPC cells. To do this, the bone marrow-
138 derived neutrophils (BMDNs) were isolated from WT C57BL/6 mice, and stimulated
139 with the NETs inducer, phorbol 12-myristate 12-acetate (PMA). Compared to the
140 vehicle control, NETs formed from PMA-stimulated murine BMDNs significantly
141 enhanced the migration (Figure 1L) and adhesion (Figure 1M) of *Rbl^{Δ/Δ}Trp53^{Δ/Δ}* NEPC
142 cells. Collectively, these data suggest that recruitment of neutrophils and NETs
143 formation promote the liver metastasis in NEPC.

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145 **5-HT secreted from NEPC potentiates NETs formation and NEPC liver metastasis.**

146 5-HT is a well-characterized neurotransmitter, produced by brain serotonergic
147 neurons, enterochromaffin cells and neuroendocrine tumors (25). Several lines of
148 evidence have shown that neuroendocrine cancer cells, such as NEPC, produce high
149 levels of 5-HT (21, 25). In support of this, we analyzed the RNA-seq data of the
150 Beltran's prostate cancer dataset(26), and found that the 5-HT biosynthesis enzyme

151 Tryptophan Hydroxylase 1 (TPH1) was markedly upregulated in NEPC compared to
152 other PCa subtypes (Supplemental Figure 1C). These findings led us to explore whether
153 NEPC-secreted 5-HT can affect NETs formation and NEPC liver metastasis.

154 To this end, we treated murine BMDNs with 5-HT, and used a potent inducer of
155 NETs, the PMA, as a positive control (27). The cell-impermeant dye SytoxGreen, which
156 can bind to DNA released by neutrophils, was stained and imaged to serve as a
157 measurement of NETs. We found that the number of NETs formed by 5-HT-stimulated
158 neutrophils was similar to that induced by PMA (Figure 2, A and B). We next collected
159 the conditioned-medium (CM) from scramble shRNA (scram-CM) and *shTph1*-
160 infected (*shTph1*-CM) *Rbl^{Δ/Δ}Trp53^{Δ/Δ}* NEPC organoids in which 5-HT production was
161 blocked to stimulate murine BMDNs respectively (Supplemental Figure 1, D and E).
162 In contrast to scram-CM, *shTph1*-CM incurred less NETs production, as evidenced by
163 the reduced percentages of SytoxGreen⁺ cells (Figure 2, C and D) and H3cit⁺
164 neutrophils (Supplemental Figure 1, F and G). To confirm whether these results were
165 applicable to the human context, we purified human peripheral blood-derived
166 neutrophils (PBDNs) and observed increased NETs formation of human PBDNs in
167 response to 5-HT stimulation (Figure 2, E and F, and Supplemental Figure 1, H and I).
168 NET-DNAs have a decondensed structure compared to intact chromatin DNAs, making
169 them more susceptible to digestion into smaller fragments by micrococcal nuclease
170 (MNase) (11). HL-60 cells can be induced to differentiate into granulocytes by N, N-
171 Dimethylformamide (DMF). Upon calcium ionophore treatment, HL-60-derived
172 granulocytes release long stretches of decondensed chromatin and form NETs (11). We
173 therefore conducted MNase digestion assay on HL-60 granulocytes and found that
174 addition of 5-HT accelerated the process of MNase-mediated DNA digestion compared
175 to the calcium ionophore treatment alone (Figure 2G). These data indicate that 5-HT

176 derived from NEPC can induce the formation of NETs.

177 For *in vivo* experiments, the scramble and sh*Tph1*-transfected *Rb1^{Δ/Δ}Trp53^{Δ/Δ}*
178 NEPC organoids were intravenously inoculated into WT C57BL/6 mice. A significantly
179 reduced liver metastatic burden was observed in the mice receiving the *Tph1*
180 knockdown (*Tph1*-KD) organoids (Figures 2, H-J). On the other hand, the attenuated
181 liver metastasis induced by *Tph1*-KD NEPC organoids was reversed upon 5-HT
182 reconstitution (Figure 2, H-J), suggesting the crucial role of 5-HT in promoting NEPC
183 liver metastasis. Co-IF staining of H3cit and MPO revealed that *Tph1*-KD in NEPC
184 organoids led to suppressed hepatic NETs formation in nearby areas of metastatic
185 cancer cells compared to scramble shRNA-transfected counterparts (Figure 2K-L).
186 Interestingly, the attenuated NETs formation (H3cit⁺MPO⁺ signal) induced by *Tph1*-
187 KD organoids was restored by 5-HT reconstitution *in vivo* (Figure 2, K and L).

188 To test whether 5-HT is also important for liver metastasis in other neuroendocrine
189 cancer types, we conducted *in vivo* experiments using the human small cell lung cancer
190 (SCLC) NCI-H82 cells (Supplemental Figure 2, A-E.) and the human medullary thyroid
191 cancer TT cells (Supplemental Figure 2, F-J). Consistent with the data from the NEPC
192 liver metastasis model, *TPHI*-KD in either NCI-H82 cells or TT cells resulted in
193 significantly suppressed liver metastasis (Supplemental Figure 2, A-C. and 2, F-H.) and
194 decreased NETs formation (Supplemental Figure 2, D and E and 2, I and J) in both
195 NCI-H82 and TT xenograft models. Collectively, *Tph1*-mediated 5-HT synthesis plays
196 an important role in promoting the liver metastasis of pleiotropic neuroendocrine
197 malignancies, including NEPC, SCLC, and medullary thyroid cancer, via facilitating
198 NETs formation.

199

200 **TGM2-mediated H3Q5ser promotes NETs formation.**

201 5-HT could either activate 5-HT receptors (HTR) or be transported into the cell
202 through the serotonin transporter (SERT) (Figure 3A). When transported into the
203 nucleus, 5-HT can covalently bind to histone catalyzed by transglutaminase 2 (TGM2),
204 leading to histone H3Q5ser modification (Figure 3A). This modification has recently
205 been shown to increase chromatin accessibility (28). Therefore, we asked whether
206 H3Q5ser modulates chromatin status in neutrophils and participates in NETs formation.
207 To this end, murine BMDNs were induced for NETs production by 5-HT, or PMA as a
208 positive control. IF staining for H3Q5ser, SytoxGreen and H3cit were conducted to
209 evaluate H3Q5ser levels and NETs formation. The results showed that 5-HT treatment
210 led to an increase in positive H3Q5ser signals with a concomitant increment of NETs
211 formation and H3cit levels in murine BMDNs (Figure 3, B-G.) and human PBDNs
212 (Supplemental Figure 3A). Next, the SERT inhibitor fluoxetine (Fluox) and the TGM2
213 inhibitor LDN-27219 (LDN) were used to block either 5-HT intake or H3Q5ser
214 modification in neutrophils (Figure 3A). IF staining data suggested that 5-HT-promoted
215 H3Q5ser modification in murine BMDNs was inhibited by either Fluox or LDN (Figure
216 3, B and C). In line with the decreased H3Q5ser levels, we also detected an evident
217 attenuation of NETs formation, as exemplified by reductions in SytoxGreen (Figure 3,
218 D and E) and H3cit (Figure 3, F and G) signals in response to either Fluox or LDN
219 treatment. We then tested whether TGM2 facilitates NET-DNA decondensation. The
220 MNase Digestion Assay revealed that suppression of TGM2 resulted in a MNase
221 hyposensitivity profile, which was indicative of a less open chromatin architecture
222 (Figure 3H). Next, we isolated murine BMDNs from a *Tgm2* knockout (*Tgm2*^{-/-}) mice.
223 Immunoblotting data revealed a concomitant decrease in H3Q5ser and H3cit
224 modifications upon the genetic ablation of *Tgm2* (Supplemental Figure 3B). Compared
225 to WT murine BMDNs, IF staining images showed that 5-HT-induced NETs formation

226 was significantly suppressed in *Tgm2*^{-/-} murine BMDNs (Figure 3, I-K.). These data
227 suggest a critical role of TGM2 in chromatin decondensation and NETs formation.

228 In addition to the 5-HT/SERT signaling, 5-HT can also bind and activate its
229 corresponding receptor (5-HT receptor, HTR) to initiate downstream signaling cascades
230 (Figure 3A). We therefore asked whether the 5-HT/HTR signaling is also involved in
231 H3Q5ser modification and/or NETs formation as well. According to previous literature,
232 HTR1B has been reported to highly expressed in neutrophils (29). We also re-analyzed
233 Beltran's clinical prostate cancer bulk RNA-seq data of NEPC liver metastasis (26),
234 and found that the HTR1B was the highest HTR at mRNA level in NEPC liver
235 metastatic biospecimens (Supplemental Figure 4A). We therefore blocked HTR1B
236 activity in murine neutrophils using HTR1B inhibitor SB224289 (Figure 3A), and
237 found that 5-HT-induced H3Q5ser modification (H3Q5ser⁺ signal) and NETs formation
238 (SytoxGreen⁺ and H3cit⁺ signals) were not significantly affected upon HTR1B
239 inhibition (Supplemental Figure 4, B-E.).

240 To further corroborate the essential role of H3Q5ser on NETs formation (Figure
241 4A), we overexpressed either HA-tagged histone H3.3-WT or H3.3-Q5A mutant in HL-
242 60 granulocytes (Figure 4B). As shown in Figure 4B, Q5A mutation of H3.3 blocked
243 the formation of H3Q5ser. Meanwhile, the H3cit deposition was markedly attenuated
244 when H3Q5ser was inactivated. IF results further showed that NETs formation was
245 significantly suppressed when the H3Q5ser modification was inhibited in the
246 H3.3(Q5A) expressing HL-60 cells in comparison to H3.3(WT)-transfected
247 counterparts (Figure 4, C-F). Furthermore, the MNase assay results demonstrated a less
248 accessible chromatin structure in H3.3(Q5A)-transfected HL-60 cells as compared to
249 that in H3.3(WT)-transfected cells (Figure 4G). Altogether, these results demonstrated
250 that TGM2-mediated H3Q5ser facilitates NETs formation.

251

252 **Genetic deletion or small molecule inhibition of TGM2 suppresses NEPC liver**
253 **metastasis.**

254 We subsequently investigated the impact of TGM2 inhibition on NEPC liver
255 metastasis. Syngeneic C57BL/6 mice were injected intravenously with *Rb1^{Δ/Δ}Trp53^{Δ/Δ}*
256 NEPC organoids. TGM2 inhibitor LDN (Figure 3A) was applied starting from 4 days
257 post inoculation. We noticed a significant reduction of liver metastasis in LDN-treated
258 mice (Figure 5, A-D). IF staining on liver sections of vehicle and LDN-treated NEPC
259 tumor-bearing mice revealed a significant decrease in H3Q5ser modifications in hepatic
260 neutrophils upon LDN (Figure 5, E and F). In accordance with repressed H3Q5ser
261 levels, H3cit modification in hepatic neutrophils was simultaneously suppressed
262 (Figure 5, G and H). To validate the role of TGM2 in driving liver metastasis in other
263 neuroendocrine cancer types, we treated the SCLC NCI-H82 cell-inoculated nude mice
264 with the TGM2 inhibitor LDN. As shown in Supplemental Figure 5, A-C, significantly
265 attenuated liver metastatic lesions were observed in LDN-treated SCLC tumor-bearing
266 mice compared to vehicle-treated counterparts. Co-IF staining of H3Q5ser/MPO and
267 H3cit/MPO revealed a significant reduction of H3Q5ser levels (Supplemental Figure 5,
268 D and E) and NETs production (Supplemental Figure 5, F and G) in liver sections upon
269 LDN treatment *in vivo*.

270 On the other hand, we employed the WT and *Tgm2^{-/-}* mice as xenograft recipients
271 for *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* NEPC organoids. Significant reductions in liver metastasis foci
272 (Supplemental Figure 6, A and B), NETs formation (Supplemental Figure 6, C and D)
273 and H3Q5ser deposition (Supplemental Figure 6, E and F) were observed in *Tgm2*
274 knockout recipient mice. Therefore, our data demonstrate a crucial role of TGM2 in
275 NEPC metastasis in the liver.

276

277 **TGM2 collaborates with PAD4 to orchestrate histone serotonylation and**
278 **citrullination.**

279 It is very interesting that H3Q5ser and H3cit display the same trend of change
280 during the formation of NETs, in response to treatment of H3Q5ser inhibitor, or upon
281 introduction of histone 3 mutations (Figure 3, B-G., Figure 4, B-F., and Figure 5, E-H.).
282 These findings, together with the approximate locations of these two modifications on
283 the histone 3 backbone (Figure 6A), led us to explore the potential crosstalk between
284 H3Q5ser and H3cit during NETs formation.

285 Next, we assessed the impact of inhibition or knockdown of respective catalytic
286 enzymes on these two histone modifications by immunoblotting. Consistent with IF
287 staining data in Figure 2, A and B and E and F, addition of 5-HT markedly promoted
288 the deposition of H3Q5ser and H3cit in HL-60 granulocytes (Figure 6B). Strikingly,
289 inhibition of TGM2 by LDN led to a marked downregulation of both H3Q5ser and
290 H3cit in HL-60 granulocytes (Figure 6B). Addition of fluoxetine resulted in a
291 simultaneous inhibition of H3Q5ser and H3cit (Figure 6B). On the other hand,
292 treatment of PAD4 inhibitor Cl-amidine also caused prominent decreases in H3cit and
293 H3Q5ser (Figure 6B). To validate this finding, we knocked down PAD4 or TGM2 in
294 HL-60 granulocytes and found that downregulation of PAD4 or TGM2 results in
295 prominent reductions of both modifications (Figure 6C). Moreover, the MNase
296 digestion assay showed that either TGM2 (Figure 6D) or PAD4 (Figure 6E) knockdown
297 suppressed the decondensed chromatin state that is required for NETs formation.
298 Therefore, these data suggested a mutually enhanced effect between TGM2-catalyzed
299 H3Q5ser and PAD4-mediated H3cit.

300 Furthermore, we used an alternative human cell line, HEK-293T cells, which

301 barely express TGM2 and PAD4, as a clean system to conduct exogenous expression
302 of HA-tagged TGM2 and FLAG-tagged PAD4 (6, 30). As shown in [Figure 6F](#), we found
303 that co-expression of PAD4 and TGM2 in HEK-293T led to a synergistic effect in
304 promoting deposition of both H3cit and H3Q5ser. In addition, we introduced enzyme
305 dead mutant of TGM2(C277S) or PAD4(C645S) into HEK-293T cells. A reduction of
306 H3cit was detected when TGM2 was inactively mutated ([Figure 6G](#)). Meanwhile, we
307 found that PAD4 inactivated mutation led to a H3Q5ser suppression ([Figure 6G](#)). Those
308 data together with the immunostaining results in [Figure 2 and 3](#) indicated that H3Q5ser
309 and H3cit are closely linked histone modifications.

310 To decipher the underlying mechanism of H3Q5ser and H3cit crosstalk, we first
311 tested whether an interaction exists between their respective enzyme PAD4 and TGM2.
312 We performed immunoprecipitations of exogenous PAD4 and TGM2 in HEK-293T
313 cells. As shown in [Figure 6, H and I](#), we detected a physical association of PAD4 and
314 TGM2 by co-immunoprecipitation (co-IP). The protein interaction of endogenous
315 PAD4 and TGM2 was also detected in HL-60 cells ([Supplemental Figure 7, A and B](#)).
316 To further decipher the physical association between PAD4 and TGM2, we subcloned
317 truncated mutations of these two proteins based on their well-characterized protein
318 domains ([Supplemental Figure 7, C and D](#)). Co-IP results revealed that the first
319 immunoglobulin-like domain (D1) of PAD4 and the third carboxy-terminal barrel
320 domain (D3) of TGM2 were required for their physical interaction ([Supplemental](#)
321 [Figure 7, E and F](#)). Moreover, we noticed that 5-HT addition further enhanced the
322 binding between PAD4 and TGM2 ([Supplemental Figure 7, G and H](#)). Additionally, we
323 performed pull-down assay using purified PAD4 and TGM2 proteins and demonstrated
324 a direct physical binding between these two enzymes ([Figures 6, J and K](#)). Together,
325 these data revealed a functional interplay between PAD4 and TGM2 that acts to

326 orchestrate histone serotonylation and citrullination.

327

328 **H3Q5ser and H3cit are mutually permissive modifications.**

329 To directly examine the putative role of H3Q5ser on H3cit modification, we
330 transduced either H3.3(WT) or H3.3(Q5A) into HEK-293T cells. As expected, the
331 mutation of the 5th glutamine residue of H3.3 to alanine residue prevented
332 serotonylation on H3 (Figure 6A and Figure 7A). This downregulation of histone
333 serotonylation led to a reduction of H3cit level (Figure 7A). In addition, we generated
334 the R2A, R8A, R17A and R2, 8, 17A combinatorial mutations of H3 to eliminate
335 citrullination at these sites (Figure 6A). As shown in Figure 7B, transfection of these
336 mutations decreased the H3cit mark in concordance with a reduced H3Q5ser deposition.
337 We investigated whether the H3.3(Q5A) or H3.3(R2,8,17A) mutant, which blocks
338 H3Q5ser or H3cit modification, affects the recruitment of each other's epigenetic writer.
339 Interestingly, H3.3(Q5A) mutation impeded the binding of both PAD4 and TGM2 to
340 H3 (Figure 7C). Similarly, we detected a deficient recruitment of both PAD4 and TGM2
341 to histone when the H3.3(R2,8,17A) mutant was introduced (Figure 7D). These data
342 suggest that H3cit and H3Q5ser are mutually permissive histone modifications.

343 A recent study reported that PAD2, another PAD family enzyme, promotes PCa
344 progression and castration resistance via Histone H3 citrullination at the 26th arginine
345 (R26) residue (H3R26cit)(31)(Figure 6A). We asked whether PAD2-mediated
346 H3R26cit affects the modulation of H3Q5ser and NETs formation. A PAD2 inhibitor,
347 AFM-30a (AFM), was used to treat murine BMDNs in the presence of 5-HT. PAD2
348 inhibition did not significantly affect 5-HT-induced NETs production (Supplemental
349 Figure 8, A and B). To further assess the putative role of H3R26cit in H3Q5ser
350 modification, we transfected HEK-293T cells with H3.3(WT) or H3.3(R26A)

351 constructs. As shown in [Supplemental Figure 8C](#), the mutation of H3.3(R26A) did not
352 affect H3Q5ser modification. Moreover, co-IP results revealed that PAD2 was not
353 physically associated with TGM2 ([Supplemental Figure 8, D and E](#)). These results
354 suggest that PAD2-catalyzed H3R26cit is not involved in H3Q5ser modification and
355 NETs formation.

356 To directly analyze the effect of H3Q5ser modification on the addition of H3cit
357 mark and *vice versa*, we performed *in vitro* histone serotonylation or citrullination
358 assays. *In vitro* catalytic reaction using purified PAD4 on H3 and H3Q5ser peptides
359 showed that serotonylation on H3Q5 substantially enhanced the PAD4-mediated H3cit
360 deposition compared to unmodified H3 ([Figure 7, E and F](#)). Concordantly, citrullination
361 on H3 also strengthened the TGM2-mediated H3Q5ser signal compared to unmodified
362 H3 ([Figure 7, G and H](#)). These results further support a mutual promoting effect
363 between the formation of H3cit and H3Q5ser modifications.

364 To determine the relationship between H3Q5ser and H3cit chromatin occupancy,
365 we conducted CUT & Tag experiments and compared the anti-H3cit and anti-H3Q5ser
366 antibody immunoprecipitated DNA sequence. As shown in [Figure 7, I-L.](#), genome-wide
367 localization analysis on HL-60 cells revealed a substantial overlap between H3Q5ser-
368 occupied and H3cit-marked genomic regions. Notably, NETs formation-related genes,
369 including *ITGB2*, *DNASE1*, *ITGAM*, *RIPK1*, *MPO*, and *AKT1*, were detected in the
370 H3Q5ser and H3cit co-occupied gene locus ([Figure 7M](#)). Together, our results suggest
371 that TGM2-catalyzed H3Q5ser and PAD4-mediated H3cit are closely linked and share
372 a substantial overlap of regulatory genomic regions on a genome-wide scale.

373

374 **Targeting SERT by fluoxetine blocks liver metastasis of NE cancers**

375 To investigate the clinical application of our findings, we applied FDA-approved

376 SERT inhibitor fluoxetine (Fluox) into *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* NEPC organoids intravenously
377 injected C57BL/6 mice. As shown in [Figure 8, A-C.](#), treatment with fluoxetine
378 effectively inhibited liver metastasis burden ([Figure 8A](#)). Mice that received fluoxetine
379 therapy displayed significant reductions in liver weight ([Figure 8B](#)) and number of
380 metastases ([Figure 8C](#)). Both H3Q5ser and H3cit staining intensities were decreased in
381 fluoxetine-treated liver metastatic lesions, suggesting that inhibition of 5-HT uptake
382 suppressed H3Q5ser ([Figure 8, D and E](#)). MPO and H3cit co-stained NETs were
383 significantly less presented in the liver metastasis sections of fluoxetine-treated group
384 ([Figure 8, F and G](#)). To further explore whether these findings identified in NEPC were
385 also applied to other neuroendocrine cancers, we performed additional experiments
386 using the SCLC NCI-H82 cells ([Supplemental Figure 9](#)) and medullary thyroid cancer
387 TT cells ([Figure 8, H-N.](#)). In line with the results of NEPC mouse model, fluoxetine
388 treatment significantly repressed NCI-H82 ([Supplemental Figure 9, A-C.](#)) and TT
389 ([Figure 8, H-J.](#)) liver metastasis *in vivo*. IF staining images demonstrated concomitant
390 reductions in both H3Q5ser and H3cit in MPO⁺ neutrophils in metastasis containing
391 liver sections in SCLC ([Supplemental Figure 9, D-G.](#)) and medullary thyroid ([Figure 8,](#)
392 [K-N.](#)) cancer models. These data supported a potential therapeutical value of the Food
393 and Drug Administration (FDA)-approved SERT inhibitor fluoxetine in blocking NETs
394 formation and preventing liver metastasis of pleiotropic NE cancers.

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401 **Discussion**

402 Here we demonstrate that 5-HT, derived from neuroendocrine cancer cells,
403 promotes liver metastasis through a crosstalk with neutrophils. 5-HT is taken up by
404 accumulating neutrophils in the liver via SERT and induces TGM2-mediated histone
405 serotonylation. In concert with PAD4-catalyzed histone citrullination, histone
406 serotonylation leads to a decondensed chromatin status in neutrophils, triggering NETs
407 formation, also named as NETosis. NETs then recruit and trap cancer cells, leading to
408 cancer metastasis ([Supplemental Figure 10, Graphical abstract](#)). Therapeutically,
409 inhibition of 5-HT intake via the SERT inhibitor fluoxetine can effectively abrogate
410 liver metastasis in preclinical mouse models ([Supplemental Figure 10, Graphical
411 abstract](#)). Therefore, in addition to the well-known role of 5-HT in nervous system, our
412 findings show that the neurotransmitter 5-HT acts as a positive regulator in facilitating
413 a pro-metastatic microenvironment in neuroendocrine cancers.

414 We elucidate the mechanism by which epigenetic modification of histone
415 serotonylation is required to promote NETs formation in a 5-HT-dependent manner.
416 Serotonylation has been implicated in different substrate proteins with biological
417 functions. Our recent work reported that serotonylation of GAPDH promotes CD8⁺ T
418 cell activation (7). In the current study, we uncover a function of 5-HT-induced and
419 TGM2-catalyzed histone serotonylation in neutrophil, a subtype of innate immune cells.
420 This conclusion is supported by multiple lines of evidence including TGM2 or SERT
421 inhibition, TGM2 mutation or knockout, and H3 (Q5A) mutation. We include three NE-
422 cancer models, NEPC, SCLC and thyroid medullary cancer in this study. Of note,
423 central nervous system cancers such as glioblastoma or nerve-infiltrating cancers also
424 produce high levels of 5-HT (32, 33). Whether a similar mechanism of 5-HT-induced
425 histone serotonylation in neutrophils and NETs formation is also involved in the

426 progression of these malignancies would be interesting to explore.

427 We uncover a reciprocally reinforcing effect between the histone modifications of
428 H3Q5ser and H3cit, and a previously unreported crosstalk between their respective
429 writers of TGM2 and PAD4. Coordinated histone modifications have been
430 demonstrated to be essential for regulating chromatin accessibility and transcriptional
431 activity (12). For example, it has been shown that Nsd1-mediated H3K36me2 co-
432 localizes with PRC2-mediated H3K27me2 and that histone methyltransferase Nsd1
433 modulates the Polycomb repressive complex 2 (PRC2) function to demarcate
434 H3K27me2 and H3K27me3 for transcriptional regulation (34, 35). In the current study,
435 another collaboration between H3Q5ser and H3cit is revealed, based on exquisite
436 biochemical experiments including TGM2 and PAD4 knockdown or enzymatically
437 dead mutations, the H3Q5ser (H3Q5A) and H3cit (H3R2,8,17A) deficient mutation, as
438 well as the immunoprecipitation, pull-down, CUT&Tag-seq, and *in vitro* histone
439 serotonylation and citrullination assays using multiple cell lines in both murine and
440 human settings. Furthermore, using *Tgm2* knockout mice and orthotopically and
441 intravenously inoculated preclinical models of NEPC, SCLC and thyroid medullary
442 cancer, our results imply that the crosstalk between H3Q5ser and H3cit is of functional
443 importance in NETs formation and cancer metastasis. Given that H3Q5ser plays a vital
444 role in neuronal differentiation and olfactory sensory processing, and that PAD4-
445 expressing neurons release citrullinated proteins in Alzheimer's disease, the crosstalk
446 between H3Q5ser and H3cit may also be relevant to nervous system functions and
447 awaits future study (6, 36, 37).

448 In summary, we find that histone serotonylation and histone citrullination are two
449 closely linked types of histone modifications in neutrophils that are orchestrated to
450 regulate chromatin decondensation, drive NETs formation, and promote liver

451 metastasis. 5-HT-driven NETs production highlights a targetable neurotransmitter-
452 immune axis in driving liver metastasis of neuroendocrine cancers. Targeting the key
453 enzyme of 5-HT biosynthesis enzyme Tph1, serotonylation enzyme TGM2 or 5-HT
454 transporter SERT are found to effectively inhibit the NETs formation and liver
455 metastasis in NEPC. Among them, the SERT inhibitor fluoxetine is an FDA-approved
456 antidepressant drug with good biosafety and bioavailability (2), suggesting a promising
457 translational value in drug repurposing. Interestingly, a recent study showed that
458 fluoxetine inhibits NEPC growth by suppressing the cell-autonomous role of 5-HT on
459 cancer cells (38). Different from this related work, our study focuses mainly on NEPC
460 liver metastasis and demonstrates that fluoxetine blocks H3Q5ser and NETs formation
461 by interfering with the effects of 5-HT on neutrophils. Therefore, fluoxetine acts as a
462 potential drug in harnessing cancer cell growth and distant metastasis of advanced
463 prostate cancer via different mechanisms.

464 Not only limited to NEPC, a high incidence of liver metastasis is commonly seen
465 in pleiotropic neuroendocrine malignancies (33, 39, 40). In addition to the three kinds
466 of tested NE cancer models including NEPC, SCLC, and medullary thyroid cancer in
467 this study, the molecular mechanism and therapeutic strategy we propose is informative
468 for a wide range of NE malignancies with such a propensity for liver metastasis.

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476 **Methods**

477 **Sex as a biological variable.**

478 Our study on prostate cancer used male mice, as it is a male-exclusive disease. For
479 experiments on small cell lung cancer and medullary thyroid cancer, male mice were
480 chosen due to their lower phenotypic variability. The relevance of these findings to
481 female mice is unknown.

482

483 **Mice.**

484 All animals used in this study were housed under humidity and temperature-
485 controlled condition with a regular light-and-dark cycle in the specific pathogen-free
486 facilities at Renji Hospital. The prostate-specific dual-depletion of *Rb1* and *Trp53*
487 transgenic mouse model *Probasin-Cre⁺; Rb1^{ff}; Trp53^{ff} (Rb1^{Δ/Δ}Trp53^{Δ/Δ})* mice were
488 obtained from the Jackson laboratory. The conventional *Tgm2*-knockout (*Tgm2^{-/-}*) mice
489 were commercially available from Cyagen Biosciences Co. Ltd (Jiangsu, China). The
490 6~8-week-old C57BL/6 mice and athymic nu/nu nude mice were purchased from
491 Shanghai SLAC laboratory animal Co.Ltd (Shanghai, China).

492

493 **Cell lines.**

494 The human granulocyte cell line HL-60 cells, human small cell lung cancer cell
495 line NCI-H82 cells, medullary thyroid carcinoma cell line TT cells, and HEK-293T
496 cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai,
497 China). The *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* NEPC murine prostate cancer organoids were established
498 and cultured as previously reported (19). All cells used in this study have been validated

499 by short tandem repeat (STR) analyses. The cells were maintained in incubators at 37°C
500 with 5% CO₂. HL-60 cells were cultured in IMDM (Gibco) with 20% FBS. NCI-H82
501 cells were cultured in RPMI-1640 (Gibco) with 10% FBS. TT and HEK-293T cells
502 were cultured in DMEM (Gibco) with 10% FBS.

503

504 **Immune analysis.**

505 In detail, the Stand Up to Cancer (SU2C) prostate cancer dataset was downloaded
506 and the gene expression matrix of liver metastases was obtained. Next, we employed
507 the quanTIseq function in Multi-omics Immuno-Oncology Biological Research (IOBR)
508 R package (<https://github.com/IOBR/IOBR>, version: 4.3.2) to analyze the infiltration
509 of immune cells in liver metastatic biospecimens from 6 patients (n = 6). The
510 enrichment of 10 immune cell subsets in liver metastasis was generated by the
511 quanTIseq function. Subsequently, the ggplot function in the ggplot2 (3.5.1) package
512 was used to generate a pie chart revealing fractions of the 10 subsets of infiltrating
513 immune cells, including B cell, dendritic cell (DC), M1-macrophage (mac-M1), M2-
514 macrophage (mac-M2), monocyte (mono), Neutrophils (Neutro), Natural killer cell
515 (NK), CD4⁺, and CD8⁺ T cells, and regulatory T cell in [Figure 1A](#). In addition, the
516 frequencies of neutrophil in adrenal, bone, liver, lymph node, and other visceral
517 metastatic sites were analyzed using the quanTIseq function to generate a violin plot
518 (vioplot R package 0.5.0) in [Figure 1B](#).

519

520 **Tumor xenograft experiments.**

521 The wild type (WT) C57BL/6 mice (6~8-week-old) were intravenously injected
522 with 2,000 *Rbl^{Δ/Δ}Trp53^{Δ/Δ}* organoid spheres. For DNase I treatment, mice were
523 intraperitoneally injected (i.p.) with DNase I at the dosage of 50 μg per mouse every
524 other day, starting from day 4 after inoculation, until the mice were sacrificed on day
525 30. For LDN-27219 treatment, mice were i.p. injected daily with 15 mg/kg LDN-27219
526 from day 4 after inoculation, and maintain the daily administration until day 30. For the
527 fluoxetine treatment, 10 mg/kg of fluoxetine was administered via oral gavage every
528 other day from day 4 after inoculation until the mice were sacrificed on day 30. The
529 nude mice (6-week-old, male) were intravenously injected with 5×10^5 human TT cells
530 or 5×10^5 human NCI-H82 cells. Nude mice were i.p. injected with 15 mg/kg LDN-
531 27219 every other day, starting from day 4 after inoculation until day 30 when the
532 tumor-bearing mice were sacrificed. 10 mg/kg of fluoxetine per mouse was
533 administrated to the *Rbl^{Δ/Δ}Trp53^{Δ/Δ}* organoid-inoculated mice via oral gavage from day
534 4 to day 30 after inoculation. Fluoxetine was dissolved in drinking water at a
535 concentration of 20 mg/L.

536

537 ***In vitro* NETs production assay.**

538 To assess NETs formation, coverslips were put into 24-well plates in advance, and
539 neutrophils were seeded in RPMI-1640 or conditioned medium (CM) at the number of
540 2.5×10^5 cells per well. 5-HT (100 μM), TGM2 inhibitor (LDN-27219, 10 μM), SERT
541 inhibitor (fluoxetine, 10 μM), PAD4 inhibitor (Cl-amidine, 100 μM) or CM were added
542 to stimulate neutrophils for 12 to 16 h. The SytoxGreen (Beyotime, C1070M) and

543 Hoechst (Invitrogen, H3570) staining were performed immediately after these
544 stimulated neutrophils were collected. Anti-H3cit, anti-H3Q5ser, and anti-MPO
545 immunostainings were performed after cells were fixed with 4% paraformaldehyde
546 (PFA). Images were captured using the Confocal Microscopy Cell Observer (ZEISS).
547 The number of H3cit⁺, H3Q5ser⁺ and MPO⁺ cells were quantified using the Image-J
548 software.

549

550 **Statistics.**

551 Data were statistically analyzed using the GraphPad 8.4.3 software. Data were
552 presented as mean ± SEM. Kaplan-Meier analysis was performed to analyze animal
553 survival, and Log-rank test was used for comparison among groups. The correlation
554 between 2 modification levels was analyzed via Spearman's correlation test. Two-tailed
555 student's *t*-tests or Mann-Whitney tests were used to determine the significance in two-
556 group experiments. One-way ANOVA tests or Kruskal-Wallis tests were used to
557 determine the significance in multiple group experiments. Data with statistical
558 significance (* P < 0.05; ** P < 0.01; *** P < 0.001) or with no statistical significance
559 (ns) were indicated in the figure panels.

560

561 **Study approval.**

562 All animal experiments were approved by the Animal Use and Care Committee at
563 Renji Hospital, Shanghai Jiao Tong University School of Medicine. The collection and
564 utilization of patient clinical samples including primary prostate tumors and liver
565 metastases were approved by the Ethics Committee of Renji Hospital, Shanghai Jiao

566 Tong University School of Medicine. Informed consents were obtained from patients
567 and donors.

568

569 **Data availability.**

570 The CUT&Tag data in this study have been deposited into the database of the
571 China National Center for Bioinformation with the accession number HRA007336.
572 Values of all data points are presented in the Supporting Data Values file. Other
573 methods were available in **Supplemental Methods and Materials.**

574

575 **Author contributions**

576 K.Y.L and Y.C.Z performed most of the experiments and contributed equally to
577 this work. G.Y.D, X.Y.C and C.X.Z conducted bioinformatic analyses and CUT&Tag
578 experiments. L.L.X and N.J assisted in data collection and biochemical assays. P.H.X,
579 H.F.Z, J.M.W, C.P.C and D.W helped with cell culture and plasmid construction. Y.Y.L,
580 L.Y.J and Y.J.S assisted in mice breeding. W.X and J.H.P provided clinical samples. W-
581 Q.G, Z-G.Z, S-H.J and P.C.Z provide important discussion, technical help and assisted
582 in manuscript preparation. H.H.Z, K.Z and K.Y.L wrote the manuscript. H.H.Z and K.Z
583 were funded. H.H.Z and K.Z supervised this study. H.H.Z conceived this study.

584

585 **Declaration of interests**

586 The authors declare no conflict of interests.

587

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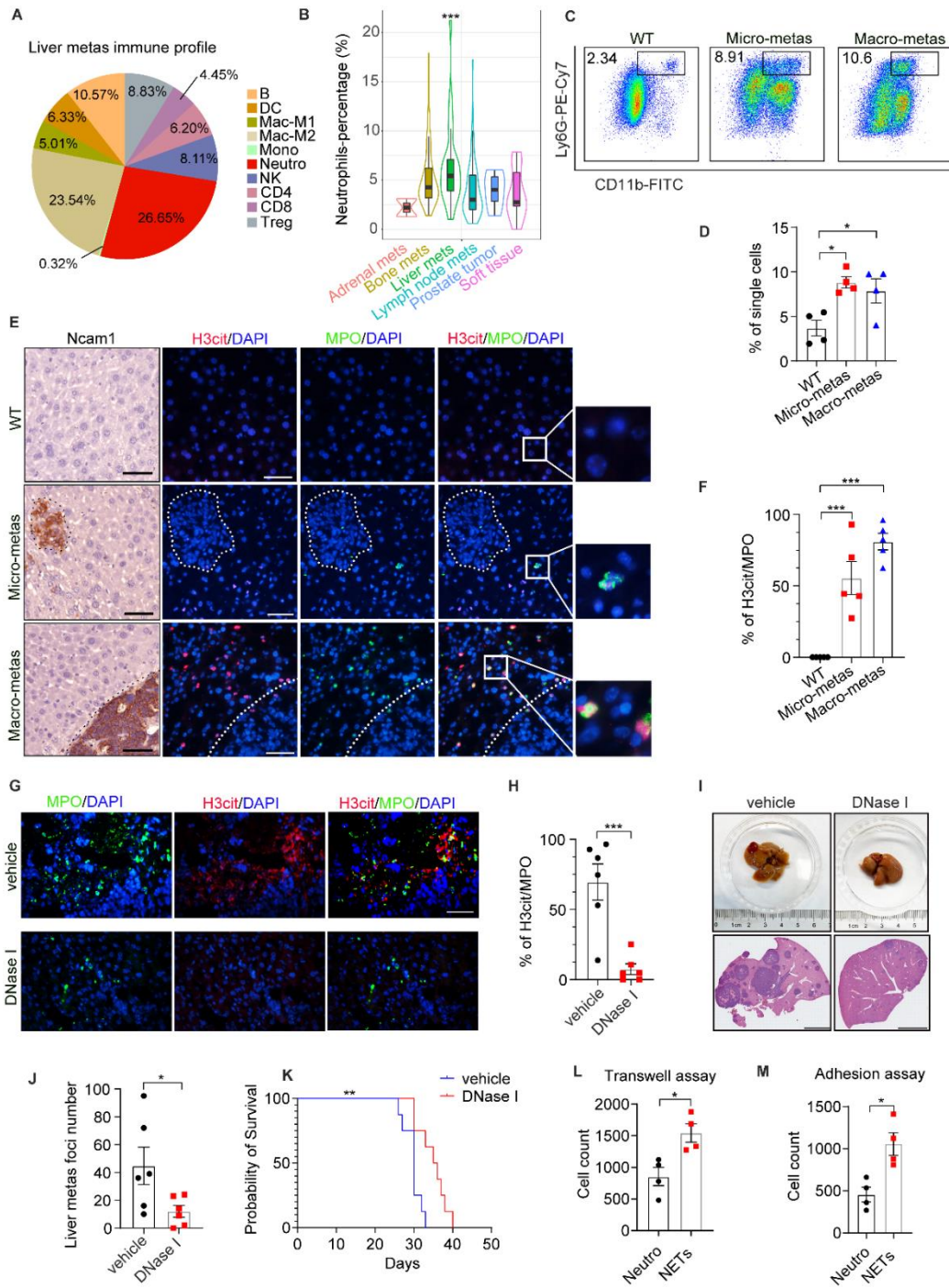
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750 **Figure 1. Neutrophils infiltration and NETs are detected in NEPC liver metastasis.**

751 **(A)** Immune cell profiles of liver metastasis based on SU2C PCa dataset (n = 26).

752 **(B)** Neutrophil is the most enriched immune cell component in liver metastasis based

753 on SU2C dataset (Adrenal mets, n = 2; Bone mets, n = 82; Liver mets, n = 26; Lymph

754 node mets, n = 79; Prostate tumor, n = 5; Soft tissue, n = 14). Data were mean \pm SEM,
755 ***P < 0.001 was assessed using One-way ANOVA followed by the Dunnett's test.

756 **(C-D)** Flow cytometric plots **(C)** and quantification **(D)** on CD11b⁺Ly6G⁺ neutrophils
757 in livers (n = 4 mice).

758 **(E-F)** Immunohistochemical (IHC), immunofluorescence (IF) staining **(E)** and
759 quantification **(F)** for NETs in liver (n = 5 mice). Scale bar = 50 μ m.

760 **(G-H)** IF staining **(G)** and quantification **(H)** on NETs (H3cit⁺) in MPO-positive
761 neutrophils in livers of vehicle and DNase-I-treated NEPC-tumor-bearing mice (n = 6
762 mice). Scale bar = 50 μ m.

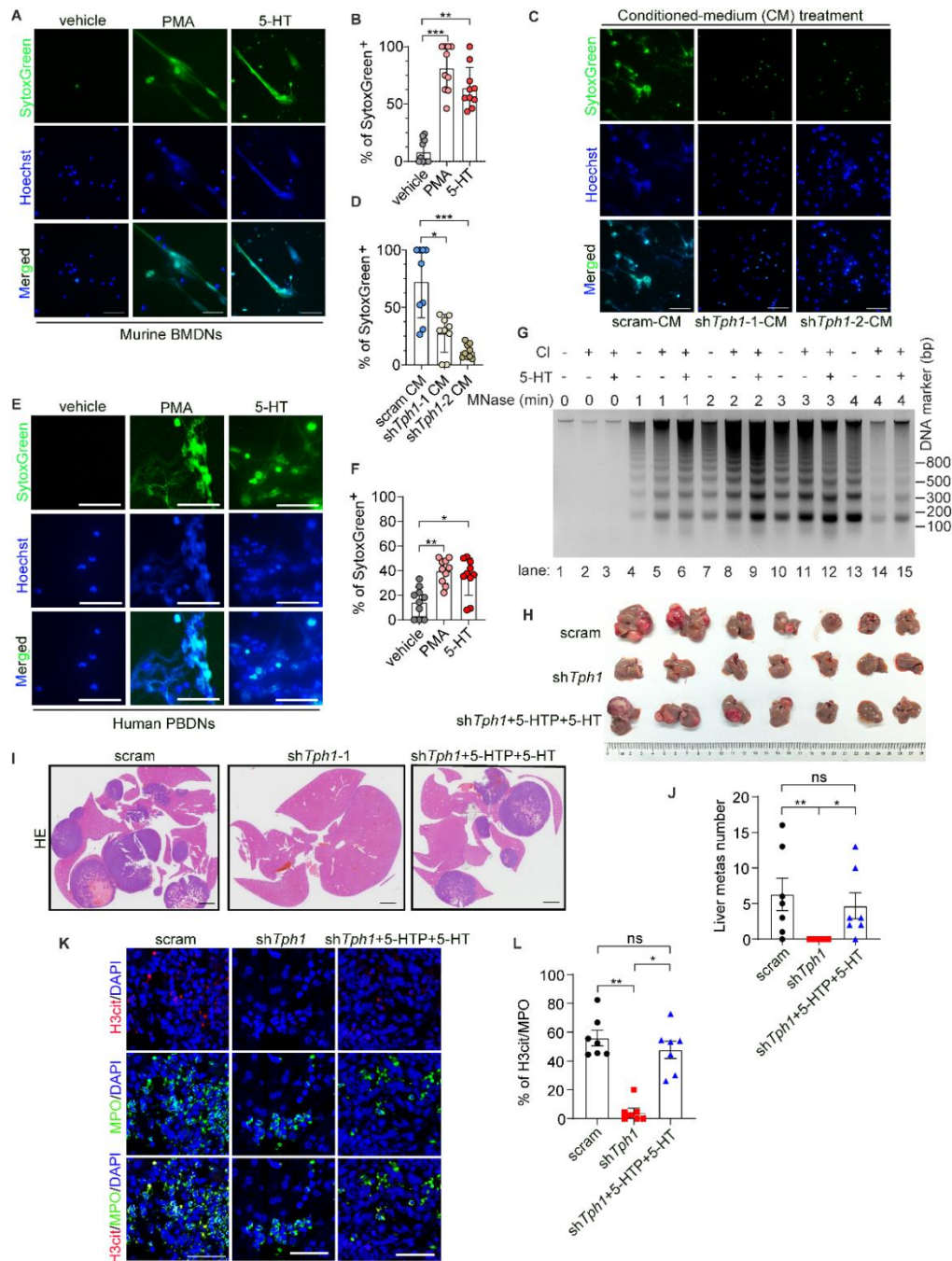
763 **(I-J)** Images **(I)** of livers from vehicle and DNase-I treated NEPC-tumor-bearing mice.
764 H&E images **(I)**, scale bars = 4 mm) and quantification **(J)** on liver metastases in
765 *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* organoid-inoculated mice via intravenous injection upon vehicle or
766 DNase-I treatment (n = 6 mice).

767 **(K)** The survival curves of vehicle and DNase-I treated NEPC-tumor-bearing mice (n
768 = 8 mice). Data were analyzed by Log-rank test. **P < 0.01.

769 **(L)** Quantification of migrated *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* PCa cells in Boyden chambers recruited
770 by murine primary neutrophils (neutro) or NETs (n = 4 biological replicates).

771 **(M)** Quantification of *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* PCa cells adhered by murine primary neutrophils
772 or NETs (n = 4 biological replicates).

773 For statistics in **(H)**, **(J)**, **(L)** and **(M)**, two-tailed student's *t*-tests were applied, and in
774 **(D)** and **(F)**, the One-way ANOVA test was used followed by Turkey's test. Data were
775 shown as mean \pm SEM, *P < 0.05 and ***P < 0.001.



776

777 **Figure 2. NEPC-derived-5-HT potentiates NETs formation and liver metastasis.**

778 (A-B) IF staining images (A) and quantification results (B) showing NETs formation
 779 of murine bone-marrow-derived neutrophils (murine BMDNs) in response to 5-HT and
 780 vehicle control (n = 10 biological replicates). Scale bars = 50 μ m. PMA induces NETs
 781 formation and serves as a positive control.

782 (C-D) IF staining images (C) and quantification results (D) showing NETs formation

783 of murine BMDNs upon the treatment of conditioned-media collected from scramble
784 and sh*Tph1*-infected *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* organoids (n = 9 biological replicates). Scale bars
785 = 50 μm.

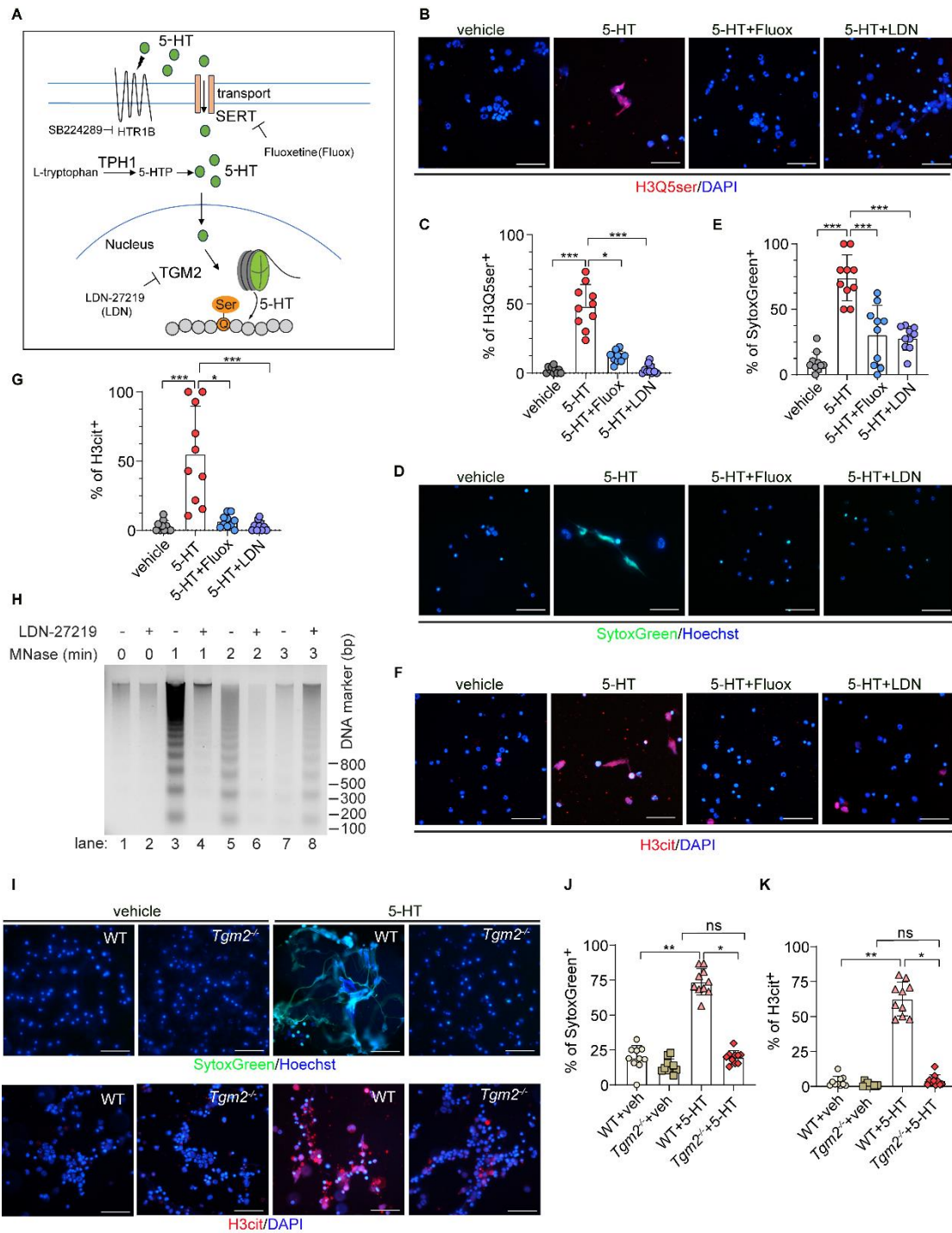
786 **(E-F)** IF images **(E)** and quantification **(F)** on NETs formation capacity of human
787 peripheral blood-derived neutrophils (PBDNs) upon 5-HT and vehicle control (n = 10
788 biological replicates). Scale bars = 50 μm.

789 **(G)** MNase digestion assay showing that addition of 5-HT induces decondensed NETs
790 chromatin in HL-60 granulocytes compared to calcium ionophore treatment.

791 **(H-J)** In vivo experiments demonstrating a reduction of liver metastatic burden **(H)** in
792 sh*Tph1*-infected as compared to scramble shRNA, and a regained liver metastatic
793 burden in 5-HTP-treated-sh*Tph1*-infected *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* organoid-implanted mice via
794 intravenous injection (n = 7 mice, each group). H&E staining **(I)** and quantification data
795 validate significantly decreased liver metastatic foci numbers in *Tph1*-knockdown
796 *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* organoid-inoculated mice and regained liver metastatic foci numbers in
797 5-HTP-treated mice **(J)**. Scale bars = 2 mm.

798 **(K-L)** IF images on liver sections **(K)** and quantification results **(L)** revealing a
799 significant decline in NETs formation in *Tph1*-knockdown *Rb1^{Δ/Δ}Trp53^{Δ/Δ}* organoid-
800 inoculated mice (n = 7 mice, each group).

801 For **B, D, F, and L**, data were assessed using Kruskal-Wallis tests followed by
802 Dunnett's tests. For **J**, data were analyzed using the One-way ANOVA followed by the
803 Tukey's test. Data were mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001. ns = no
804 significance.



805

806 **Figure 3. 5-HT induces histone serotonylation in neutrophils during NETs**
 807 **formation.**

808 **(A)** A schematic chart showing the 5-HT-activated 5-HT/HTR signaling, SERT-
 809 mediated 5-HT intake, the TPH1-catalyzed 5-HT-biosynthesis, and the TGM2-
 810 catalyzed H3Q5ser.

811 **(B-C)** IF staining images **(B)** and quantification results **(C)** demonstrate that 5-HT
812 addition promotes H3Q5ser modification in murine BMDNs, and SERT inhibitor
813 fluoxetine (Fluox) and TGM2 inhibitor LDN-27219 (LDN) compromise 5-HT-induced
814 H3Q5Ser (n = 10 biological replicates). Scale bars = 50 μ m.

815 **(D-G)** IF staining results and quantification data reveal that fluoxetine and LDN-27219
816 abrogate 5-HT-induced NETs formation, as reflected by SytoxGreen **(D and E)** and
817 H3cit **(F and G)** signals (n = 10 biological replicates per experiment). Scale bars = 50
818 μ m.

819 **(H)** The MNase digestion assay showing that LDN-27219-treatment of HL-60
820 granulocytes leads to a delayed chromatin decondensation than vehicle controlment
821 upon the stimulation of calcium ionophore.

822 **(I-K)** The *Tgm2*^{-/-} mice-derived BMDNs showing significantly reduced NETs
823 formation upon 5-HT stimulation, as exemplified by reduced SytoxGreen **(I and J)** and
824 H3cit **(I and K)** signals, comparing to WT mice-derived BMDNs (n = 10 biological
825 replicates per experiment). Scale bars = 50 μ m.

826 For statistics in **C, G, J and K**, the Kruskal-Wallis tests were performed followed by
827 Dunnett's tests. Data in **(E)** were assessed using the One-way ANOVA followed by the
828 Tukey's test. Data were mean \pm SEM, *P < 0.05, **P < 0.01 and ***P < 0.001. ns = no
829 significance.

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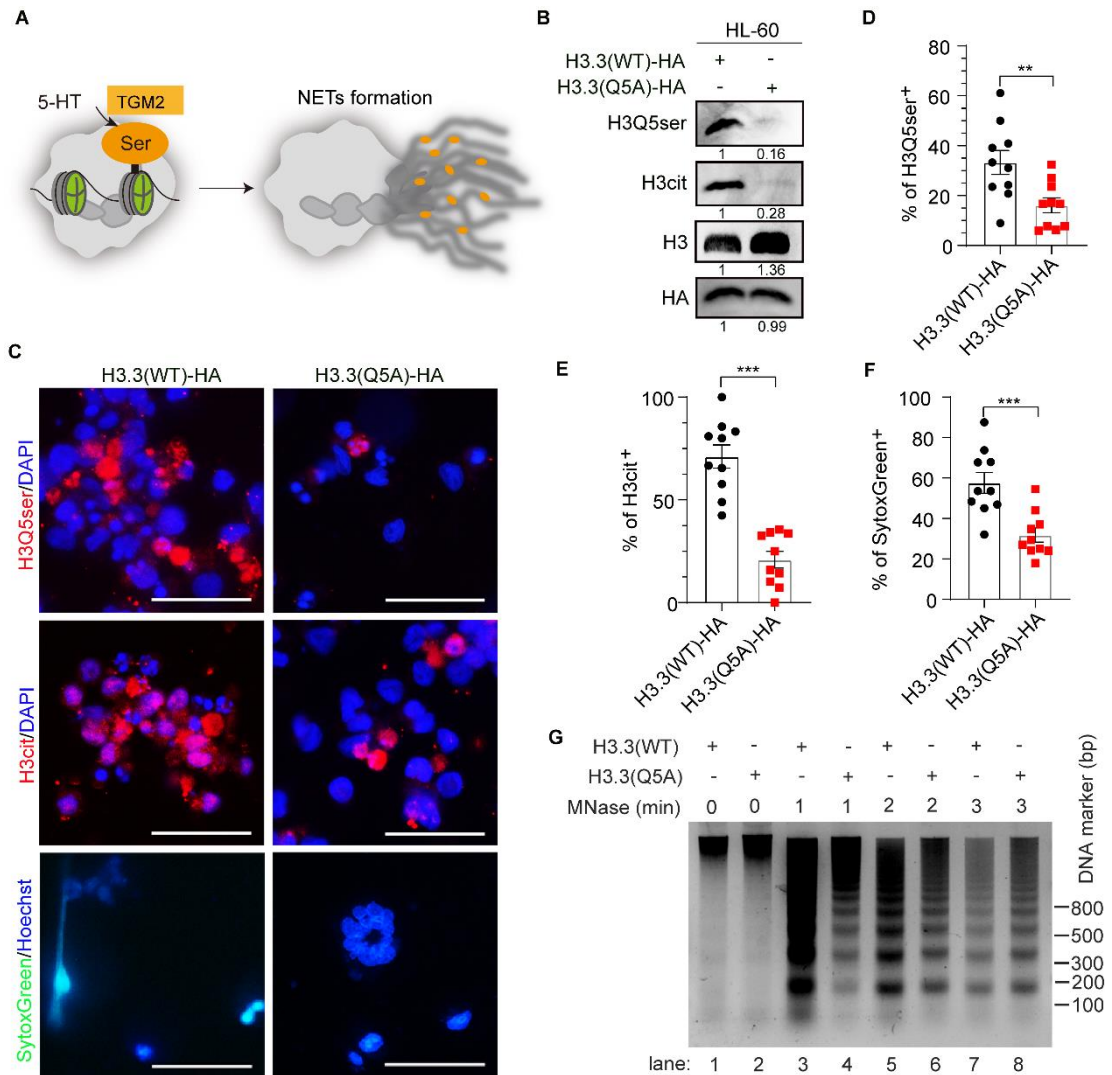


Figure 4. H3Q5Ser modification promotes NETs formation.

(A) A schematic diagram showing that 5-HT-mediated histone seronylation promotes a decondensed chromatin state and facilitates NETs formation.

(B) Immunoblotting results demonstrate reduced levels of H3Q5Ser and H3Cit modifications in H3.3(Q5A) mutant as compared to H3.3-WT-transfected HL-60 granulocytes.

(C-F) IF staining images (C) and quantification data (D-F) showing reductions in H3Q5ser (D) and NETs formation in H3.3(Q5A) mutant-transfected HL-60 granulocytes cells, as reflected by decreased H3cit (E) and SytoxGreen (F) signals (n

843 = 10 biological replicates per experiment). In C, Scale bars = 50 μ m.

844 **(G)** The MNase assay data reveal that chromatin in H3.3(Q5A)-transfected HL-60
845 granulocytes is less accessible than that in H3.3(WT)-transfected cells in response to
846 calcium ionophore induction.

847 For statistics in **(D)**, **(E)** and **(F)**, two-tailed student's *t*-tests were used and data were
848 mean \pm SEM, **P < 0.01 and ***P < 0.001.

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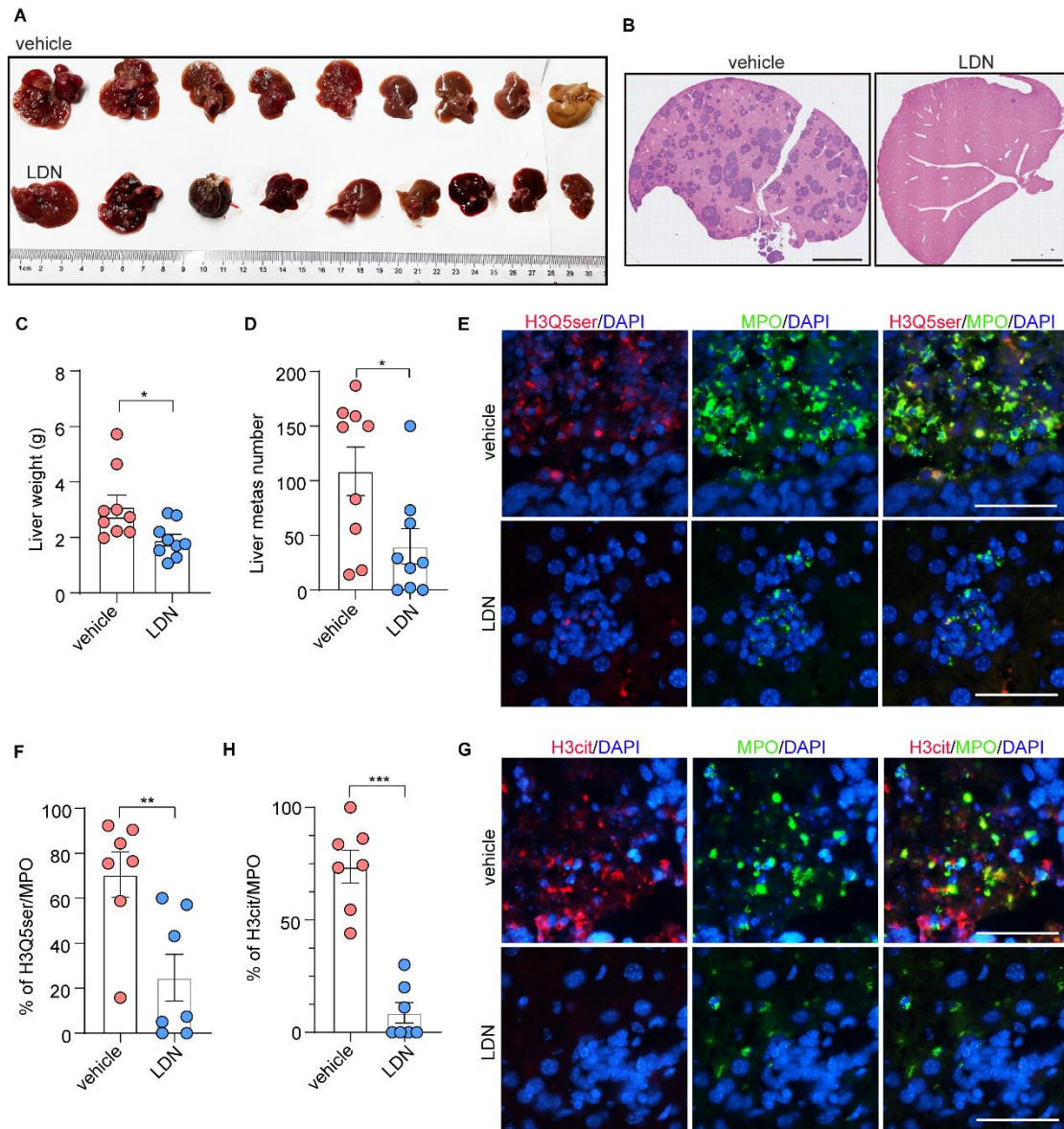
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856 **Figure 5. TGM2 inhibition abrogates NETs formation and liver metastasis in**
857 **NEPC mouse models.**

858 **(A)** TGM2 inhibitor LDN-27219 suppresses liver metastatic burden in *Rb1^{Δ/Δ}Trp53^{Δ/Δ}*-
859 inoculated mice via intravenous injection (n = 9 mice, each group).

860 **(B)** H&E staining assay showing reduced liver metastatic foci in response to the
861 treatment of LDN-27219. Scale bars = 4 mm.

862 **(C-D)** Quantifications on liver weights **(C)** and liver metastatic foci numbers **(D)** in
863 *Rb1^{Δ/Δ}Trp53^{Δ/Δ}*-inoculated mice upon the treatment of vehicle and LDN-27219 (n = 9

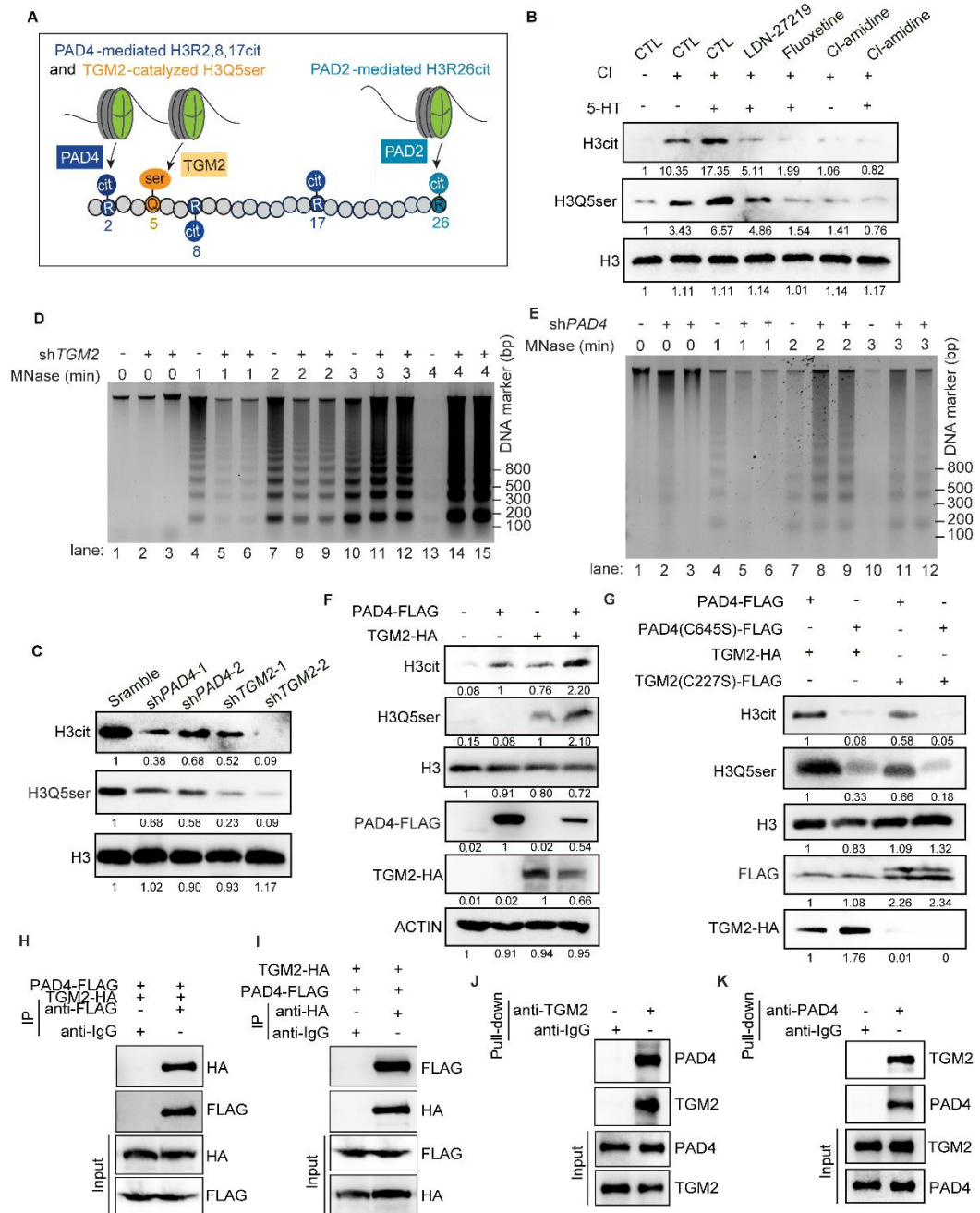
864 mice, each group).

865 **(E-F)** IF staining images **(E)** and quantification results **(F)** showing decreased histone
866 serotonylation (H3Q5ser⁺) in hepatic neutrophil (MPO⁺) in *Rbl^{Δ/Δ}Trp53^{Δ/Δ}*-inoculated
867 mice upon the treatment of vehicle and LDN-27219 (n = 7 biological replicates). Scale
868 bars = 50 μm.

869 **(G-H)** IF images **(G)** and quantifications **(H)** on neutrophil (MPO⁺)-derived NETs
870 (H3cit) in *Rbl^{Δ/Δ}Trp53^{Δ/Δ}*-inoculated mice in response to vehicle and LDN-27219 (n =
871 7 biological replicates). Scale bars = 50 μm.

872 For **(C)**, **(D)**, **(F)** and **(H)**, the Mann-Whitney tests were applied and data were shown
873 as mean ± SEM, *P < 0.05, **P < 0.01 and ***P < 0.001.

874



875

876 **Figure 6. TGM2 collaborates with PAD4 to coordinate histone seronylation and**
 877 **citryllination.**

878 **(A)** A schematic diagram showing proximal locations of TGM2-catalyzed H3Q5ser and
 879 PAD4-mediated H3R2,8,17cit and PAD2-promoted H3R26cit on Histone H3.

880 **(B)** Immunoblotting assays demonstrate increased H3cit and H3Q5ser levels in HL-60-
 881 derived granulocytes upon 5-HT stimulation.

882 **(C)** Immunoblotting data reveal reductions in H3cit and H3Q5ser modifications in
883 either sh*PAD4* or sh*TGM2*-HL-60-cells compared to scramble shRNA-transfected cells.

884 **(D-E)** The MNase assay data demonstrate that knockdown of either *TGM2* **(D)** or *PAD4*
885 **(E)** in HL60 cells attenuates calcium ionophore-induced chromatin decondensation.

886 **(F)** Dual expression of HA-tagged TGM2 and Flag-tagged PAD4 in HEK-293T cells
887 showing elevated levels of both H3cit and H3Q5ser modifications.

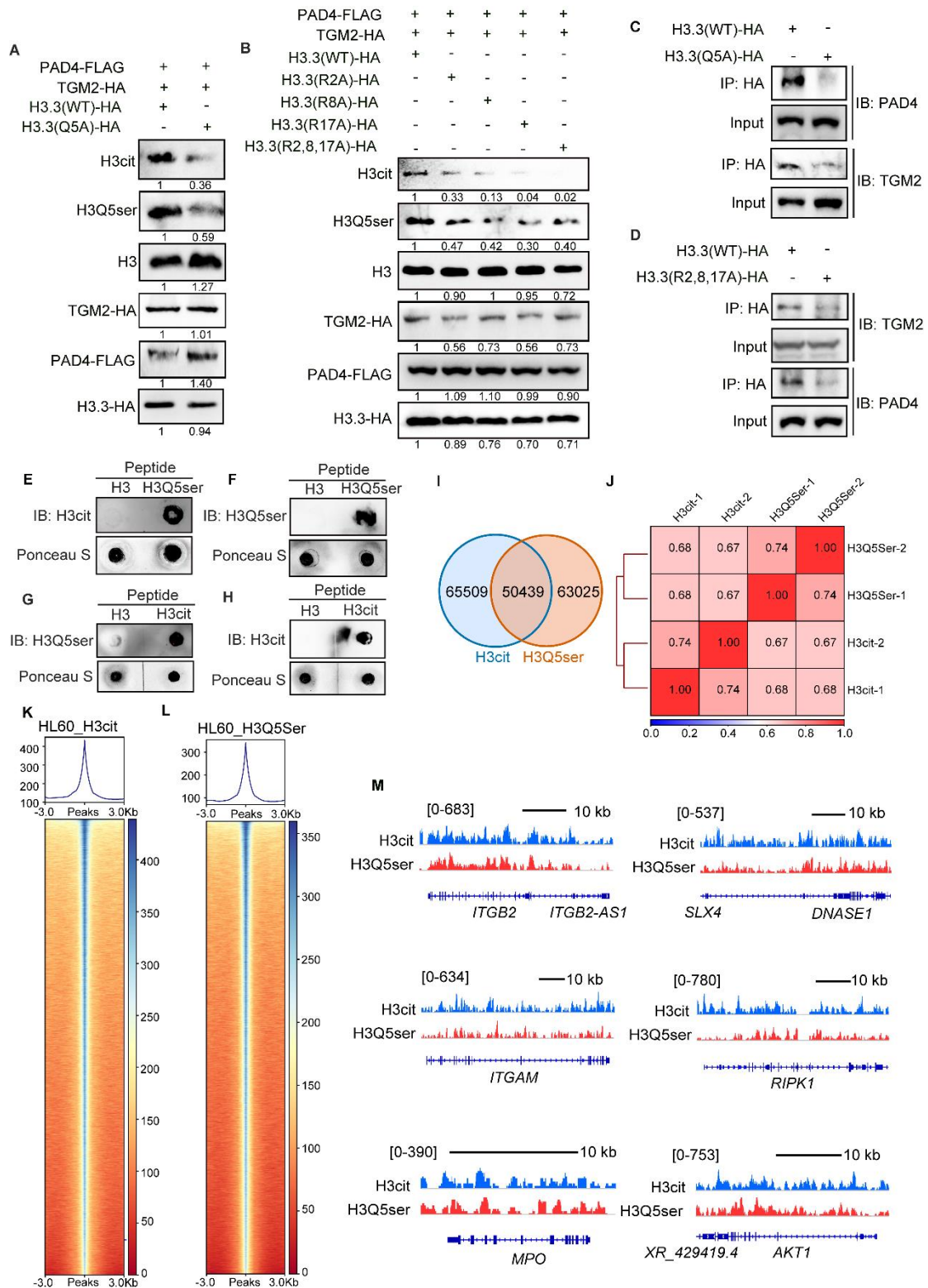
888 **(G)** Expression of enzyme-dead mutant of TGM2-C277S and PAD4-C645S in HEK-
889 293T cells abrogates their synergistic effect in enhancing H3cit and H3Q5ser
890 depositions.

891 **(H-I)** Co-immunoprecipitation (co-IP) assay shows an exogenous interaction between
892 TGM2 and PAD4 by IP Flag-tagged PAD4 **(H)** and HA-tagged TGM2 **(I)** in HEK-293T
893 cells.

894 **(J-K)** Pulldown assay demonstrates the protein-protein association between TGM2 and
895 PAD4 *in vitro*.

896

897



898

899 **Figure 7. H3Q5ser and H3cit are mutually enhanced and share chromatin**
 900 **occupancy on a genome-wide scale.**

901 **(A)** Immunoblotting assay demonstrates that the H3.3(Q5A) mutant leads to deficient

902 H3Q5ser modification and a reduced H3cit level.

903 **(B)** Immunoblotting data show that either H3.3(R2A), H3.3(R8A), H3.3(R17A)
904 mutants alone or in combination H3.3(R2,8,17A) result in deficient H3cit modification
905 and a repressed H3Q5ser level.

906 **(C-D)** Mutant H3.3(Q5A) **(C)** or H3.3(R2,8,17A) **(D)** attenuates the recruitment of each
907 other's epigenetic writer, as exemplified by deficient binding of either PAD4 **(C)** or
908 TGM2 **(D)** to histone H3.

909 **(E-F)** In vitro catalytic assay showing that H3Q5ser-modified peptides enhance PAD4-
910 mediated H3cit compared to unmodified H3 peptide control.

911 **(G-H)** In vitro catalytic assay revealing that H3cit-modified peptides promote TGM2-
912 mediated H3Q5ser compared to unmodified H3 peptide control.

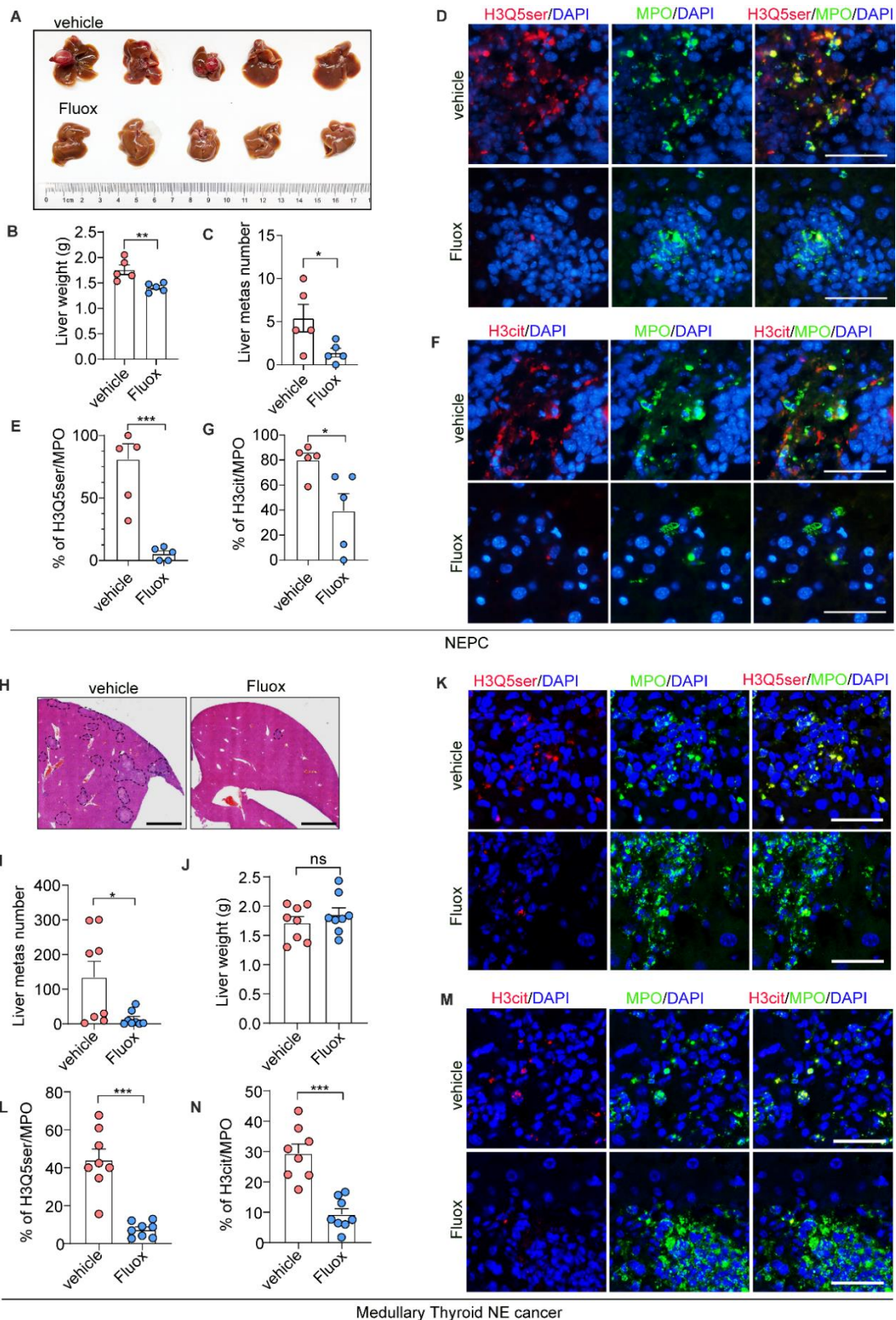
913 **(I)** Venn diagrams show the overlapped H3cit and H3Q5ser peaks in HL-60
914 granulocytes from CUT&Tag sequencing data (2 independent experiments).

915 **(J)** Heatmap showing the genome-wide Spearman's correlation between the H3Q5ser
916 and H3cit modifications (2 independent experiments).

917 **(K-L)** Heatmaps of H3cit **(K)** and H3Q5ser **(L)** peaks are showed on a genome-wide
918 scale in HL-60 granulocytes (2 independent experiments).

919 **(M)** CUT&Tag profiles of co-occupied NETs related genes including *ITGB2*, *DNASE1*,
920 *ITGAM*, *RIPK1*, *MPO*, and *AKT1* of H3cit and H3Q5ser modifications in HL-60
921 granulocytes (2 independent experiments).

922



923

924 **Figure 8. SERT inhibitor fluoxetine represses NETs formation and liver metastasis**

925 **in NE-cancers.**

926 **(A-C)** SERT inhibitor fluoxetine suppresses liver metastasis **(A)** in *Rb1^{Δ/Δ}Trp53^{Δ/Δ}*.

927 intravenously inoculated mice, as exemplified by decreased liver weights **(B)** and
928 metastasis foci numbers **(C)**, comparing to vehicle-treated ones (n = 5 mice, each group).
929 **(D-E)** IF staining images **(D)** and quantification results **(E)** showing decreased histone
930 serotonylation (H3Q5ser⁺) in hepatic neutrophils (MPO⁺) in *Rbl^{Δ/Δ}Trp53^{Δ/Δ}*-inoculated
931 mice upon the treatment of fluoxetine as compared to vehicle (n = 5 mice, each group).
932 Scale bars = 1.5 mm.

933 **(F-G)** IF images **(F)** and quantifications **(G)** on neutrophil (MPO⁺)-derived NETs
934 (H3Cit) in *Rbl^{Δ/Δ}Trp53^{Δ/Δ}*-inoculated mice in response to vehicle and fluoxetine (n = 5
935 mice, each group). Scale bars = 50 μm.

936 **(H-J)** H&E staining images **(H)** and quantification results showing decreased
937 metastasis foci numbers **(I)** in TT-cells-inoculated mice via intravenous injection upon
938 the treatment of fluoxetine, comparing to vehicle-treated counterparts (n = 8 mice, each
939 group). Scale bars = 1 mm.

940 **(K-L)** IF staining images **(K)** and quantification results **(L)** showing decreased
941 H3Q5ser⁺ in MPO⁺ in TT-inoculated mice upon the treatment of fluoxetine as compared
942 to vehicle (n = 8 mice, each group). Scale bars = 50 μm.

943 **(M-N)** IF images **(M)** and quantifications on NETs **(N)** in TT-inoculated mice in
944 response to vehicle and fluoxetine (n = 8 mice, each group). Scale bars = 50 μm.

945 For statistics in **(B)**, **(C)**, **(E)**, **(J)**, **(L)** and **(N)**, two-tailed student's *t*-tests were used
946 and data were mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001, and ns = no
947 significance. For **(G)** and **(I)**, data were mean ± SEM, *P < 0.05 was assessed using
948 Mann-Whitney tests.